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Application of a Two-Dimensional Electrophoresis Method to the Systematic Study of Land Snails of Subgenus *Luchuphaedusa* from Southwestern Japan Islands

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Abstract. A land snail, *Tyrannophaedusa (Luchuphaedusa) ophidoon* (family Clausiliidae), is classified into two types, large and small forms, according to shell size. Using a two-dimensional electrophoresis method, we compared the total protein components of the large form with seven members of *Luchuphaedusa* and three species of different subgenera of the same genus, and obtained the similarity ranging from 0.989 to 0.667. The similarity between the large form and *T. (Decolliphaedusa) bilabrata* was the lowest. Two species of the other subgenus, *Nesiophaedusa*, were very similar to the large form. The differences were very small between two specimens of the large form. The small form of *T. (L.) ophidoon* showed large differences from the large form. Thus, the relationship of the large and small forms is considered to be either closely related interspecific populations or distantly differentiated intraspecific populations, by comparing our result with available data obtained previously. Based on values of the similarity obtained, we discuss possible colonization patterns in *Luchuphaedusa*.

Introduction

Luchuphaedusa is a group of Japanese clausiliid snails originally described by Pilsbry (1901) as "section Luchuphaedusa." Kuroda (1963) placed this group in one of three subgenera in the genus *Tyrannophaedusa*. Based on Pilsbry's original description, this group has distinctive morphological characteristics in the shell aperture, the clausilium, and the plicae. In particular, the clausilium is very peculiar and unlike those of any closely related Clausiliid group.

Luchuphaedusa comprises eight species (two having one subspecies) and lives mainly on the Nansei Islands between the Pacific Ocean and the East China Sea (Kuroda, 1963). Only one species inhabits a small area of the Kyushu mainland; two species live on the western islands of Kyushu. Their distribution is distinctive and incompatible with biogeographical boundaries suggested by distributions of terrestrial vertebrates, avians, spiders, insects, and other land snails (Tokuda, 1978). Therefore, it is intriguing to investigate how these slow-moving animals have expanded their habitats, established their unique distribution, and speciated. There is no study so far concerning patterns of their expansion of distribution and speciation and the phylogenetic relationships of species belonging to this group.

T. (L.) ophidoon among *Luchuphaedusa* was originally described by Pilsbry (1905) as making up a distinctive "section Oophaedusa." This species has a peculiar shell shape which does not taper at the summit, while shells of other species are shaped like those of ordinary clausiliids. The species has been classified as the large or small form according to shell size (Pilsbry, 1905; Minato, 1985). Although Minato (1985) concluded that these two forms were conspecific, recent studies show that they are considerably different judging from some anatomical characters and allozyme variation (Ueshima, in prep.). Thus, these two forms are very ambiguous in their species identities. Therefore, it is intriguing to examine their characters from different viewpoints and to discuss the phylogenetic position of *T. (L.) ophidoon* in subgenus

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Luchuphaedusa and taxonomic status of the two forms of this species relating to other species.

We have established a basis for elucidating the evolutionary process of *Luchuphaedusa* by analyzing protein constituents of whole bodies using two-dimensional electrophoresis. The method permits the simultaneous analysis of many characters (Aquadro and Avise, 1981; Miyazaki *et al.*, 1987) and avoids the need to consider intraspecific variation. This is in contrast to one-dimensional electrophoresis which analyzes variable proteins, *i.e.*, enzymes (Ayala *et al.*, 1974; Avise, 1975). We calculate the similarity among species based on two-dimensional electrophoretic patterns and discuss a radiation pattern and the relationships between these land snails.

Materials and Methods

Samples

Species used in this study are listed below. Three species belonging to two different subgenera of *Tyrannophaedusa*, including type species of respective subgenera, were also examined.

(i) *T. (L.) azumai azumai* and *T. (L.) ophidoon* (large and small forms) from Shimo Koshiki-jima Is.

(ii) *T. (L.) nesiothauma* and *T. (L.) oshimae* from Amami O-shima Is.

(iii) *T. (Nesiophaedusa) okinoerabuensis* from Okinoerabu-shima Is.

(iv) *T. (L.) callistochila*, *T. (L.) inclyta*, and *T. (N.) bernardi* from Okinawa Is.

(v) *T. (Decolliphaedusa) bilabrata* from Sho-o-cho, Okayama, Honshu.

The system of classification was according to Kuroda (1963), although some authors treat *Luchuphaedusa*, *Nesiophaedusa*, and *Decolliphaedusa* as full genera. *Nesiophaedusa*, consisting of two species, is endemic to Nansei Islands and is similar to *Luchuphaedusa* in general shell characteristics (Pilsbry, 1901). *Decolliphaedusa* is widely distributed in the southwestern area of Japan.

T. (L.) ophidoon is classified according to shell and anatomical characteristics into large and small forms. A recent study revealed that the large form was composed of two divergent populations inhabiting northern and southern parts of Shimo Koshiki-jima Island (Ueshima, in prep.). Thus, we used only the large form specimens from the southern part of the island to avoid confusion.

A distribution of members of *Luchuphaedusa* and *Nesiophaedusa* and localities where specimens were obtained are shown on the map in Figure 1.

Electrophoresis

Whole bodies of land snails, removed from their shells, were used for electrophoresis. One pair of specimens was used for each comparison.

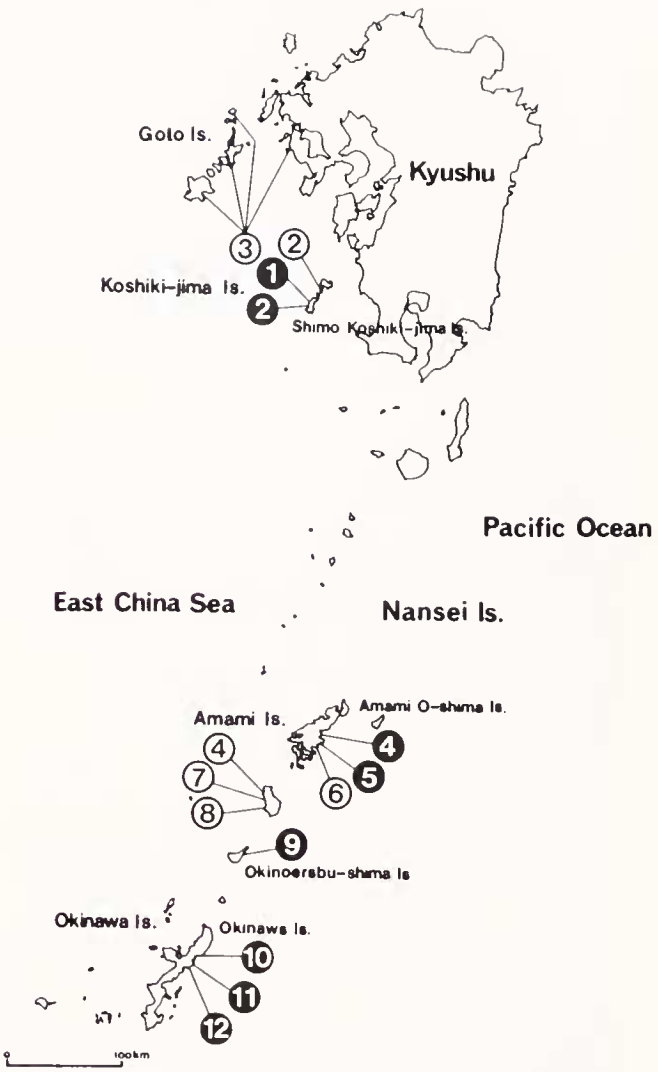

Figure 1. Map of southwestern Japan showing a distribution of members of *Luchuphaedusa* and *Nesiophaedusa* and localities of specimens used in this study. White numbers in closed circles represent islands where specimens for this study were obtained. Black numbers in open circles represent other areas in which members of *Luchuphaedusa* inhabit. 1, The large and small forms of *Tyrannophaedusa (Luchuphaedusa) ophidoon*; 2, *T. (L.) azumai azumai*; 3, *T. (L.) azumai uma*; 4, *T. (L.) nesiothauma*, 5, *T. (L.) oshimae*; 6, *T. (L.) mima mima*; 7, *T. (L.) mima tokunoshimana*; 8, *T. (L.) degenerata*; 9, *T. (Nesiophaedusa) okinoerabuensis*; 10, *T. (N.) bernardi*; 11, *T. (L.) callistochila*; 12, *T. (L.) inclyta*. *T. (Decolliphaedusa) bilabrata* is not included.

Sample solubilization and two-dimensional electrophoresis were carried out as described previously (Hirabayashi, 1981; Hirabayashi *et al.*, 1983; Oh-Ishi and Hirabayashi, 1988). In brief, the whole body was homogenized thoroughly in 20 volumes of an extraction medium containing 8 *M* guanidine HCl, which prevents protease activity completely. The homogenate was dialyzed against 5 *M* urea and 1 *M* thiourea and centrifuged

at 60,000 × *g* for 45 min. The supernatant (80 μl or 120 μl) was subjected to the first dimension isoelectric focusing with agarose gels for 12,500 V·h. The second dimension SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970), at 30 mA when the bromophenol tracking dye was in the stacking gel of 3% acrylamide and at 60 mA until the dye reached the lower end of the running gel of a concentration gradient of 12–20% acrylamide. After electrophoresis, proteins were stained by Coomassie brilliant blue as described by Stephano *et al.* (1986).

Analysis

A method for comparison between two specimens has been described (Miyazaki, 1987). For comparison of two-dimensional electrophoresis patterns, we used a triplet method in which two different samples to be compared (each 80 μl) and their mixture (60 + 60 μl) were focused and electrophoresed at the same time. One set composed of three patterns was examined on photographs. The overlapping of protein spots was confirmed on the mixture pattern after it was presumed from comparison between two individual patterns. A close examination of the mixture pattern is indispensable to identify subtle differences in positions of spots and to judge precisely the overlapping of spots, although only individual patterns were used for comparison by some authors (Ohnishi *et al.*, 1983a; Ohnishi *et al.*, 1983b; Williams, 1984).

In most cases we used the large form as a standard counterpart for comparison, because its taxonomic position relative to others is especially intriguing. About two to four hundred protein spots were examined on each electrophoretic pattern and the similarity was calculated as described by Aquadro and Avise (1981).

Results

Two typical sets for comparison between two-dimensional electrophoresis patterns are represented in Figure 2. Triplet patterns on the left are for investigating variation between different individuals of the large form of *Tyrannophaedusa (Luchuphaedusa) ophidoon* (Fig. 2a, b, c). Those on the right are for comparison between the large form of *T. (L.) ophidoon* and *T. (Nesiophaedusa) bernardi* (Fig. 2d, e, f). The pattern of *T. (N.) bernardi* (f in Fig. 2) is very different from that of the large form (d in Fig. 2). The patterns from different specimens of the large form from the same locality (a, c in Fig. 2) are very similar, showing almost no variation between individuals. Middle patterns (b, e in Fig. 2) of each set are derived from mixtures of two different samples.

The similarity was calculated according to the formula: $F = 2N_{xy}/(N_x + N_y)$, where F is the similarity between specimens x and y, N_{xy} is the number of spots shared by x and y, and N_x and N_y are the numbers of spots scored for x and y, respectively (Aquadro and Avise, 1981). The result of calculations is shown in Table 1. The similarity value between different specimens of the large form was very large (0.989), while the value between the large and small forms was smaller (0.913), suggesting that the large and small forms are definitely different populations. The result supports that *T. (Decolliphaedusa) bilabrata* is most divergent from *Luchuphaedusa*, because the similarity between *T. (D.) bilabrata* and the large form was the lowest (0.667) of all combinations examined. However, two species of other subgenus *Nesiophaedusa*, *T. (N.) okinoerabuensis* and *T. (N.) bernardi*, were more similar to the large form rather than some members of *Luchuphaedusa*, such as *T. (L.) inclyta*.

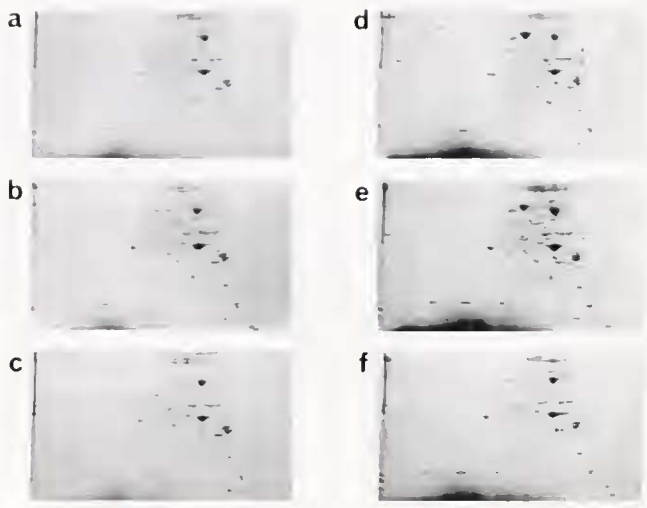

Figure 2. Two-dimensional electrophoresis patterns of whole bodies of land snails in genus *Tyrannophaedusa*. Electrophoresis was carried out as described previously (Hirabayashi, 1981; Oh-Ishi and Hirabayashi, 1988). Triplet patterns for investigating variation between different individuals of the large form of *T. (Luchuphaedusa) ophidoon* are represented on the left (a–c). Patterns from two different specimens of the same locality (a and c) and their mixture pattern (b) are shown. Triplet patterns for comparison between the large form of *T. (L.) ophidoon* and *T. (Nesiophaedusa) bernardi* are represented on the right (d–f), where d is the pattern of the large form, f is that of *T. (N.) bernardi*, e is that of their mixture.

Schematic drawing of a distribution of similarity values is shown in Figure 3. There were three large gaps of values; the first is between the large and small forms (0.076), the second between the small form and *T. (L.) azumai azumai* (0.067), and the third between *T. (L.) inclyta* and *T. (D.) bilabrata* (0.069). One may suppose that the large and small forms which live on Shimo Koshiki-jima Island (Fig. 1) are divergent from *T. (L.) azu-*

Table I

Similarity among land snails of Tyrannophaedusa

Combination		Similarity
	T. (L.) ophidoon (large form)	
a	vs. *T. (L.) ophidoon* (large form)	0.989
b	vs. *T. (L.) ophidoon* (small form)	0.913
c	vs. *T. (L.) azumai azumai*	0.846
d	vs. *T. (L.) nesiothauma*	0.834
e	vs. *T. (N.) okinoerabuensis*	0.824
f	vs. *T. (N.) bernardi*	0.795
g	vs. *T. (L.) callistochila*	0.768
h	vs. *T. (L.) oshimae*	0.766
i	vs. *T. (L.) inclyta*	0.736
j	vs. *T. (D.) bilabrata*	0.667
	T. (L.) callistochila	
k	vs. *T. (L.) oshimae*	0.878
l	vs. *T. (N.) okinoerabuensis*	0.833

Similarity is calculated according to Aquadro and Avise (1981). L., subgenus *Luchuphaedusa*; N., subgenus *Nesiophaedusa*; D., subgenus *Decolliphaedusa*.

mai azumai of this island, supporting a distinctive position of *T. (L.) ophidoon* as previously described by Pilsbry (1901). However, it should not be concluded unconditionally, because we do not compare directly the small form with *T. (L.) azumai azumai*. Similarly we cannot infer that *T. (L.) callistochila* and *T. (L.) oshimae* are very similar, simply because they were positioned so closely in Figure 3 (the upper line). Therefore, we made the direct comparison between *T. (L.) callistochila* and *T. (L.) oshimae* to learn whether they are closely similar or not. The result gave the largest similarity value (0.875) among values between different species (Table I), meaning that they are very similar as expected from their close positions in Figure 3 (the upper line). This situation is also supported by direct comparison between *T. (L.) callistochila* and *T. (N.)* okinoerabuensis, because they were positioned more distantly (Fig. 3, the upper line) and had a value of lower similarity (0.833) than *T. (L.) callistochila* and *T. (L.) oshimae*. The relationships of *oshimae* and *okinoerabuensis* to *callistochila* are also shown in Figure 3 (the lower line).

Discussion

Table I shows that the large form of *Tyrannophaedusa (Luchuphaedusa) ophidoon* is most similar to different specimens of the same form (similarity 0.989) and most different from species of another subgenus, *T. (Decolliphaedusa) bilabrata* (similarity 0.667). Therefore, relationships, which are considered to be most closely related or most differentiated, are demonstrated in the similarity values. This suggests that the two-dimensional electrophoresis method is suitable for systematic analysis and supports our previous conclusion that this method provides a valuable tool for systematics (Miyazaki *et al.*, 1987).

The method presents a debatable issue that species of the other subgenus, *Nesiophaedusa*, are more similar to the large form than some members of *Luchuphaedusa*. As reported by Pilsbry (1901), the general characters of shells are similar between *Nesiophaedusa* and *Luchuphaedusa*. We found no morphological differences except the clausilium. Therefore, *Luchuphaedusa* may be paraphyletic, since some members of *Luchuphaedusa* are less similar to the other member in this group than the members of the group *Nesiophaedusa*. From these considerations, we propose that *Nesiophaedusa* should be united with *Luchuphaedusa* as one subgenus. This is supported by our result that the similarity value of 0.833 is obtained between *T. (N.) okinoerabuensis* and *T. (L.) callistochila*, the type species of *Luchuphaedusa*. This value corresponds to those (0.824 and 0.795) among two species of *Nesiophaedusa* and the large form of *T. (L.) ophidoon* (Table I).

T. (L.) nesiothauma has a distinctive aperture by which it can be distinguished from other *Luchuphaedusa* species. But the result (Table I) shows that it is very similar to the large form of *T. (L.) ophidoon*. Therefore, it is necessary to examine how similar this species is to other members of *Luchuphaedusa* and to reconsider the taxonomic significance of its aperture difference.

Luchuphaedusa shows the peculiar distribution from Nansei Islands to a small area of the Kyushu mainland, which is not interrupted by biogeographical boundaries suggested by the data about other animals (Tokuda, 1978). Thus its radiation pattern is especially intriguing. Our result reveals a correlation between the similarity values and the arrangement of islands. Islands are located in the order of Amami O-shima Is., Okinoerabushima Is., and Okinawa Is. toward south from Shimo Koshiki-jima Is. (Fig. 1). The large form of *T. (L.) ophidoon* and two members which are most similar to that form inhabit Shimo Koshiki-jima Is. (Fig. 3, a, b, c). *T.*

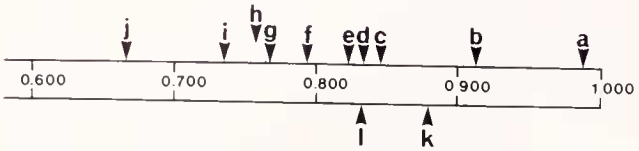

Figure 3. Schematic drawing of a distribution of similarity values among land snails. The similarity values to the large form of *Tyrannophaedusa (Luchuphaedusa) ophidoon*, which is a standard counterpart for comparison, are shown on the upper line, and those of *T. (L.) oshimae* and *T. (Nesiophaedusa) okinoerabuensis* to *T. (L.) callistochila* on the lower line. Letters correspond with those in Table I.

Table II

Distribution of the similarity values between intraspecific or interspecific populations

	Mouse		Fruit fly		Land snail	
Similarity	Intra	Inter	Intra	Inter	Intra	Inter
1.000–0.981			4		1	
0.980–0.961			1	2		
0.960–0.941	1		1	3		
0.940–0.921				6		
0.920–0.901	1			1	1*	
0.900–0.881			1	6		
0.880–0.861				5		1
0.860–0.841		1		6		1
0.840–0.821				8		3
0.820–0.801		1		2		

Figures represent the numbers of pairs which show the similarity within the range given in the left column. Intra and Inter indicate intraspecific and interspecific populations, respectively.

* Represents the position of the similarity between the large and small forms of *T. (L.) ophidoon*. The data on mice is from Aquadro and Avise (1981) and that on fruit flies (*Drosophila montium* species subgroup) is from Ohnishi *et al.* (1983b). Intraspecific populations were not found in ranges below 0.800.

(L.) nesiothauma, of which the similarity to the large form is higher next to the two above mentioned members, is an inhabitant of Amami O-shima Is. (Fig. 3, d). *T. (N.) okinoerabuensis* in Okinoerabu-shima Is. is less similar to the large form than *T. (L.) nesiothauma* (Fig. 3, e). *T. (L.) callistochila*, *T. (L.) inclyta*, and *T. (N.) bernardi*, which have the smaller similarity values to the large form, live on Okinawa Is. (Fig. 3, f, g, i). Therefore, the values show that the more remote the island is from Shimo Koshiki-jima Is., the lower similarity the species living on it has. This may be accounted for by sequential colonization of a single ancestor from either Shimo Koshiki-jima Is. or Okinawa Is., and by radiation within each island. *T. (L.) oshimae* is the only exception (Fig. 3, h) in this interpretation of radiation of these species. This species lives on Amami O-shima Is., but has significantly lower similarity than *T. (L.) nesiothauma*. After the main current of radiation was over, its ancestor may have come from some other island. The presumptive donor island is possibly Okinawa Is., because the species has the similarity value close to those of other members on this island. It is remarkably similar to *T. (L.) callistochila* in the protein constituent (Table I) and shell morphology.

The last problem concerns the taxonomic positions of the small and large forms of *T. (L.) ophidoon*. Their shell shapes are so peculiar that Pilsbry (1905) referred them to "section Oophaedusa" and included them as a single species. However, they are different to each other not only in shell size but also in anatomical and biochemical characteristics (Minato, 1985; Ueshima, in prep.). Our result also shows that they constitute considerably different populations, since there is a large gap (0.076) between the two forms (Fig. 3, the upper line). Therefore, it is questionable whether they should be included in the same species or not. When our result is compared with those reported by others using the two-dimensional electrophoresis method (Table II), the relationship between the large and small forms corresponds to that of closely related interspecific or distantly differentiated intraspecific populations. Data suggesting reproductive isolation between these two forms is under study of their allozyme variation (Ueshima, in prep.). Since available data from two-dimensional electrophoresis is insufficient, and since classification systems currently used are probably unique to different taxa, we can only assume taxonomic positions of organisms examined. Collection of data from other organisms by the two-dimensional electrophoresis method and investigation on other characteristics of the two forms are now being carried out to decide more precisely their taxonomic positions.

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